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Akt and its phosphorylation in nucleus accumbens mediate heroin-seeking behavior induced by cues in rats

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Abstract

Akt is initially identified as one of the downstream targets of phosphatidylinositol-3 kinase (PI3K) and is involved in morphine reward and tolerance. However, whether phospholyration of Akt (p-Akt) mediates heroin relapse remains unclear. Here, we aimed to explore the role of p-Akt in the nucleus accumbens (NAc) in cue-induced heroin-seeking behaviors after withdrawal. First, rats were trained to self-administer heroin for 14 days, after which we assessed heroin-seeking behaviors induced by a context cue (CC) or by discrete conditioned cues (CS) after 1 day or 14 days of withdrawal. We found that the active responses induced by CC or CS after 14 days of withdrawal were higher than those after 1 day of withdrawal. Meanwhile, the expression of p-Akt in the NAc was also greatest when rats were exposed to the CS after 14 days of withdrawal. Additionally, a microinjection of LY294002, an inhibitor of PI3K, into the NAc inhibited the CS-induced heroin-seeking behaviors after 14 days of withdrawal, paralleling the decreased levels of p-Akt in the NAc. Finally, Akt1 or β-arrestin 2 was downregulated via a lentiviral injection to assess the effect on heroin seeking after 14 days of withdrawal. CS-induced heroin-seeking behavior was inhibited by downregulation of Akt1, but not β-arrestin 2, in the NAc. These data demonstrate that Akt phosphorylation in the NAc may play an important role in the incubation of heroin-seeking behavior, suggesting that the PI3K/Akt pathways may be involved in the process of heroin relapse and addiction.

KEYWORDS
drug seeking, heroin, incubation, nucleus accumbens, phosphatidylinositol-3 kinase

1 | INTRODUCTION

Relapse into heroin use after prolonged abstinence is usually induced by exposure to drug-related cues that are associated with previous heroin reward. In rodent models, heroin-seeking behavior persists for over 2 months[1] and progressively increases during the first 2 weeks after withdrawal,[2,3] also known as the heroin incubation of craving. This incubation of heroin-seeking behavior is critically associated with neuroadaptations in the mesocorticolimbic dopamine system after chronic heroin use and subsequent withdrawal.[4] However, the molecular mechanisms underlying the incubation of heroin seeking remain largely unknown.

Huaqiang Zhu and Dingding Zhuang contributed equally to this work.

The serine/threonine protein kinase Akt, also termed protein kinase B, is an intracellular signal molecule initially identified as one of the downstream targets of phosphatidylinositol-3 kinase (PI3K).[5] Phosphorylation of Akt (p-Akt) by PI3K can subsequently interact with downstream effectors and modulate various cellular processes, including growth, survival, differentiation, and glucose metabolism. There are two phosphorylation sites of Akt; serine 473 residue is activated in a PI3K-dependent pattern, while threonine 308 residue can also be activated by other signaling pathways, such as cAMP/PKA way.[6] Akt has also been found to be regulated by addictive drugs within the mesocorticolimbic dopamine system that consists of cell bodies in the ventral tegmental area (VTA) which project to many forebrain areas, including the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), and amygdala. The p-Akt-473 levels in the NAc of rats increased by acute morphine administration but significantly decreased after chronic morphine administration.[7] It has been found that the decreased size of VTA dopamine neurons and diminished morphine reward after chronic morphine administration is mediated by the downregulation of brain-derived neurotrophic factor (BDNF)/the insulin receptor substrate 2 (IRS2)/p-Akt-473 signaling pathway in the VTA.[8] Recent evidence has revealed that the Akt pathway is involved in synaptic and structural neuroadaptations induced by addictive drugs. For example, cocaine self-administration increased the tropomyosin-related kinase B (TrkB)-dependent p-Akt protein levels in the NAc.[9] Furthermore, the Akt/mammalian target of rapamycin (mTOR) signaling pathway in the NAc is critically involved in the bidirectional synaptic plasticity after acute or chronic cocaine administration.[10] Methamphetamine-induced behavioral sensitization required increased levels of the NMDA Receptor 2B (GluN2B)-PP2A-Akt cascade in the dorsal striatum.[11] Nicotine exposure during adolescence induces the profound upregulation of the Akt- glycogen synthase kinase 3(GSK-3) signaling pathways directly within the NAc.[12] Furthermore, the p-Akt level in the NAc is reduced when rats are re-exposed to the cues associated cocaine memories in conditioned place preference model.[13] However, the mechanisms underlying the regulation of p-Akt in the incubation of heroin seeking after withdrawal or cues-induced heroin-seeking behavior remains largely unknown.

Increased dopamine (DA) neurotransmission arising from the administration of amphetamine or from the lack of DA transporter results in the inactivation of Akt in the mouse striatum (including ventral NAc and dorsal caudate putamen).[14] Another study has indicated that striatal dopamine D2 receptors exert their action in a cAMP-independent manner by promoting the formation of a signaling complex composed of Akt, protein phosphatase-2A (PP2A), and β-arrestin 2. The formation of this complex then leads to the inactivation of Akt after the dephosphorylation of its regulatory threonine 308 residue by PP2A.[15] The inactivation of Akt, in turn, results in the activation of GSK-3, which eventually contributes to the activation of the transcription factor cAMP response element binding protein (CREB) and production of dopamine-associated behaviors.[16]

The present study was first designed to characterize the expression of p-Akt in the NAc of rats during the incubation of heroin-seeking behavior induced by contextual cue (CC) or discrete conditioned cues (CS) after withdrawal. Furthermore, LY294002, an inhibitor of PI3K, was microinjected into the NAc to observe its effects on CS-induced heroin seeking after withdrawal. Finally, small interference RNA (siRNA) lentivirus of Akt1 or β-arrestin 2 was microinjected into the NAc to explore the possible interaction of Akt1 and β-arrestin 2 in the NAc on heroin-seeking behavior.

2 | EXPERIMENTAL PROCEDURES

2.1 | Subjects

Male Sprague–Dawley rats (250–300 g, Experimental Animal Center of Zhejiang Province, China) were housed in a temperature-controlled, ventilated colony room with a reversed 12-h light/dark cycle (lights onset 19:00 h, offset 07:00 h). *Ad libitum* food and water was provided in the home cage. Rats were acclimated to the environment for 1 week before experiments. The study design was approved by the Ethics Committee of Laboratory Animal Use and Care at Ningbo University, and all experiments were conducted during the dark period according to the specifications of the National Institute of Health Guide for the Care and Use of Laboratory Animals (Eighth edition).

2.2 | Drugs and construction of siRNA lentivirus

Heroin (diacetylmorphine HCl) was obtained from the National Institute of Forensic Science (Beijing, China). The Akt inhibitor LY294002 was purchased from Tocris, MO, USA, and dissolved in artificial cerebrospinal fluid (aCSF). Small hairpin RNA (shRNA) targeting Akt1 or β-arrestin 2 was constructed and synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). The rat Akt1 eukaryotic expression plasmid GP-Akt1 was constructed using the GP-GFP Vector containing a multiple cloning site for the insertion of shRNA constructs to be driven by an upstream H1 promoter and a downstream cytomegalovirus promoter-GFP (marker gene). The sequences of the shRNAs used in the present study were as follows: Akt1 (5′-GCACCTTTATTGGCTACAAGG-3′) and β-arrestin 2 (5′-GCAACTCAAGCACGAAGACAC -3′).

2.3 | Intravenous surgery

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and implanted with chronically indwelling intravenous catheters, as described previously.[17] A silicon catheter (Silastic; length 3.5 cm, 0.5-mm inner diameter, 0.94-mm outer diameter) was inserted into the right external jugular, and the other end of the catheter (10 cm, PE20) was passed subcutaneously through an incision on the back of the body, where it then entered the custom-made fluid connector fixed to a jacket. The catheter was flushed daily with 0.3-ml saline containing penicillin B (20,000 units) and heparin (5 units) to prevent bacterial infection and maintain catheter patency. Following surgery, rats were allowed 7 days to recover prior to behavioral training.

2.4 | Heroin self-administration

After recovery from surgery, the animals were placed in operant chambers from Med Associates Inc. (ENV-114 M, Saint Albans, VT) for daily 4-h heroin self-administration training sessions. Operant chambers were equipped with two infrared beam nose-poke apertures on the same panel and located 5 cm above the floor of the box, those nose pokes had a green LED light inside each hole. Heroin was delivered through Tygon tubing attached to a syringe pump at a speed of 1.2 ml/min using a 5-ml syringe. A computer-assisted system was used to control experimental procedure using a MED Associates interface and running self-programmed software written in Borland Delphi 6.0. Every session was begun with a blue light inside the active nose-poke hole. The rat received a single heroin infusion (0.2 mg/ml, 0.05 mg/kg in each infusion) following the completion of an active nose poke under the FR1 schedule. Each infusion was paired with a 20-s illumination of the house light in combination with the noise of the infusion pump, which therefore served as the discrete conditioned cues (CS) paired with the drug infusion. A timeout period was imposed for 20 s, and the light inside the active nose-poke hole was turned off, during which a response had no programmed consequences but was still recorded. Illumination of the blue light in the active nose-poke hole signaled the end of the 20-s timeout period. The number of the inactive nose poke touched was recorded, but this had no programmed consequences. All rats were trained for 14 consecutive days to reach stable response activity levels.

2.5 | Intracranial surgery and microinjection

After the last self-administration session, the rats were placed in the home chambers and underwent a spontaneous withdrawal session. In the withdrawal session, the rats did not have access to heroin, the heroin self-administration CC, or the heroin-associated CS. Next, all rats were divided into three groups in order to undergo different experiments. The first group of rats was tested for heroin-seeking behavior induced by CC or CS after different periods of withdrawal. The second group of rats was divided into three groups (eight rats per group) for intracranial surgery. These rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and positioned into a stereotaxic apparatus (Stoelting 51,950, USA). Guide cannulas, consisting of 24-gauge thin-walled stainless steel tubing (RWD Life Science Co., China), were implanted bilaterally 0.5 mm above the NAc (+1.4 mm anteroposterior, ±2.0 mm mediolateral, and −6.6 mm dorsoventral).[18] Guide cannulas were secured with stainless steel screws and dental cement, and rats were allowed at least 7 days to recover before heroin-seeking behavioral testing. The last group of rats was divided into three groups (seven rats per group) for lentivirus vector microinjection. These rats were also anesthetized with sodium pentobarbital and positioned in a stereotaxic apparatus. Microinjection needles were bilaterally placed into NAc (+1.4 mm anteroposterior, ±2.0 mm mediolateral, and −7.1 mm dorsoventral), and 0.5 μl of virus per side was injected over 5 min; thus, the microinjection speed was 0.1 μl/min. The needles were left in place for an additional 5 min to allow for the diffusion of virus particles away from the injection site.

2.6 | Heroin-seeking behavior

After the completion of withdrawal for different days, all rats were replaced in the operant chambers for the assessment of their heroin-seeking behaviors induced by the CC or CS. In the CC test, there was no light and the infusion pump noise or heroin infusion. The CS test began with a 5-s light cue that previously predicted the drug's availability and a house light that was previously associated with heroin infusion, after which each active nose-poke response resulted in another presentation of the CS but no heroin infusion. Active and inactive nose pokes during this testing phase of heroin-seeking behavior were recorded and added up.

2.6.1 | Effects of time duration on heroin-seeking behavior induced by a CC or CS

Four groups (eight per group) of rats were tested for heroin-seeking behavior induced by CC or CS after different durations of withdrawal. Following 1 day of withdrawal, two groups of rats were replaced in the operant chambers for 2 h to assess heroin-seeking behaviors induced by the CC or CS. The other two groups were tested after 14 days of withdrawal.

2.6.2 | Effects of LY294002 on CS-induced heroin-seeking behavior

Three groups of rats that had recovered from intracranial surgery were tested for heroin-seeking behaviors after 14 days of withdrawal. Prior to testing, plugs were removed, and bilateral infusion cannulas (31 gauge) were inserted, extending 0.5 mm beyond the tip of the guide cannulas. Rats were microinjected with LY294002 (2 or 5 mM) or vehicle (aCSF) into the NAc using a microinjection pump (MD-1001, Bioanalytical System Inc., IN) at a volume of 0.5 μl over 5 min. Fifteen minutes after completion of the microinjection, all rats were tested 2 h thereafter for heroin-seeking behaviors induced by the CS.

2.6.3 | Effects of β-arrestin 2 or Akt1 RNA interference on CS-induced heroin-seeking behaviors

Three groups of rats (n = 7) that had received a lentivirus microinjection were tested for heroin-seeking behaviors after 14 days of withdrawal. The negative control (NC), β-arrestin 2, or Akt1 lentivirus was expressed for 10–14 days. All rats were then tested heroin-seeking behaviors induced by the CS for 2 h. After testing, three rats per group were used for western blot analysis, and the other four rats per group were used for the histology.

2.7 | Western blot analysis

Rats were killed immediately after heroin-seeking testing. The rats were deeply anesthetized with pentobarbital (80 mg/kg, i.p.) and decapitated. The NAc tissues were isolated from the removed brain by gross dissection, and extracts were prepared from an individual rat. Western blots were carried out according to standard methods. Briefly, the NAc tissues were directly lysed in sodium dodecyl sulfate sample buffer and incubated at 95°C for 10 min before being loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel. The protein concentration was determined before being loaded onto gel and then 50-μg protein per sample was loaded onto each track. Proteins were separated by electrophoresis and then transferred to a nitrocellulose membrane (BioRad, Hercules, California, USA). The membrane was blocked in 5% milk-TBST and probed with Akt or p-Akt-308 or p-Akt-473 antibody (9272s or 9275s or 9271s, Cell Signaling Technology Inc., USA) and p-CREB antibody (sc-7978-R, Santa Cruz Biotechnology Inc., USA) (all at a 1:1000 dilution ratio), all in 3% milk-TBST, and reacted with the horseradish peroxidase-conjugated secondary antibody (1:2000) in 3% milk-TBST. Immunoreactive protein bands were detected by enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) on an X-ray film or scanned by the Odyssey Imaging System (LI-COR Biosciences, Lincoln, USA), and immunoblots were evaluated by integrating densitometry using GeneSnap and GeneTools (Chemigenius Gel Documentation System, Syngene, Cambridge, UK). β-Actin (4967s, Cell Signaling Technology Inc., USA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sc-365062, Santa Cruz Biotechnology Inc., USA) served as an internal protein control (1:2000).

2.8 | Histology

Once all testing was completed, the rats that had been microinjected with the lentivirus were anesthetized and transcardially perfused with saline, followed by a 4% polyformalin solution. The brains were sectioned on a Cryostat Microtome (Leica CM1850, Germany) in the coronal plane at a thickness of 30 μm. Lentiviral expression was observed by fluorescent microscopy (Olympus BX 53, Japan), and the localization of injection sites was mapped onto a schematic diagram of the rat brain.[18]

2.9 | Statistical analysis

Total nose-poke responses during behavioral testing were assessed using a two-way ANOVA followed by Bonferroni post-tests over the active and inactive nose pokes. The expressions of p-Akt and p-CREB protein were analyzed using a one-way ANOVA followed by Bonferroni post hoc comparisons between group means. Data are presented as means ± SEM. Statistical analysis was performed using SPSS 16.0 (SPSS, Inc.) and GraphPad Prism 8.0 (GraphPad Software, Inc.). $P < 0.05$ or $P < 0.01$ was considered to indicate a statistically significant difference.

3 | RESULTS

3.1 | Establishment of the heroin self-administration model

Figure 1 revealed similar findings to those that we had previously described.[19] The active pokes and heroin infusions of rats reached a stable high level within 14 days of heroin self-administration training. In contrast, the inactive pokes of rats were still at a low level after

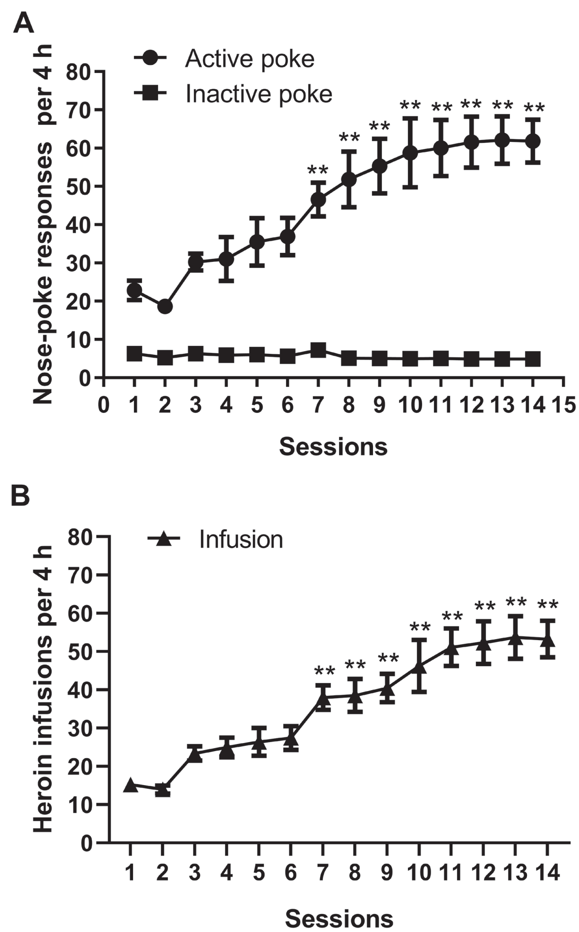

FIGURE 1 Effects of 14 days of training on the number of pokes and infusions during heroin self-administration in rats. (A) The active pokes but not the inactive pokes at days 7–14 increased significantly when compared to those at day 1, $^{**}P < 0.01$, compared with the active pokes at day 1, $n = 24$. (B) The heroin infusions at days 7–14 increased significantly when compared to those at day 1, $^{**}P < 0.01$, compared with the heroin infusions at day 1, $n = 24$

14 days of training. Two-way repeated measures ANOVA for active pokes and inactive pokes during 14 days showed a significant main effect for day ($F_{(13,\ 644)}$ = 12.66, P < 0.001), pokes ($F_{(1,\ 644)}$ = 1,197, P < 0.001), and an interaction of day × pokes ($F_{(13,\ 644)}$ = 14.07, P < 0.001), with significant increase in active pokes on days 7–14 when compared to those at day 1 (P < 0.01, respectively) but no significant differences in inactive pokes across 14 days (Figure 1A). One-way ANOVA revealed a significant main effect for day on the heroin infusions ($F_{(13,\ 322)}$ = 15.61, P < 0.001). *Post-hoc* Bonferroni comparisons revealed that the heroin infusions on days 7–14 were significantly increased when compared to those on day 1 (P < 0.01, respectively, Figure 1B).

3.2 | P-Akt expression in the NAc exposed to a CC or CS after 1 day or 14 days of withdrawal

Following the self-administration sessions, rats were assessed for the number of nose pokes induced by CC or CS after 1 day or 14 days of withdrawal. As illustrated in Figure 2A, a two-way ANOVA revealed a significant main effect for treatment ($F_{(3,\ 56)}$ = 158.9, P < 0.001), pokes ($F_{(1,\ 56)}$ = 628.6, P < 0.001), and an interaction of treatment × pokes ($F_{(3,\ 56)}$ = 135.1, P < 0.001). *Post-hoc* Bonferroni comparisons revealed that the active pokes induced by either the CC or CS after 14 days of withdrawal were significantly increased when compared to those after 1 day of withdrawal (P < 0.01, respectively). Additionally, the active pokes induced by CS were significantly increased when compared to those induced by CC not only after 1 day but also after 14 days of withdrawal (P < 0.01, respectively). There was no difference in the inactive pokes of all groups.

Figure 2B illustrated the expression of p-Akt-473 in the NAc of rats after the heroin-seeking test induced by a CC or CS following 1 day or 14 days of withdrawal. A one-way ANOVA revealed a significant main effect of the treatment on the expression of p-Akt-473 ($F_{(3,8)}$ = 264.88, P < 0.001). *Post-hoc* Bonferroni comparisons revealed that the expression of p-Akt-473 induced by either the CC or CS after 14 days of withdrawal was significantly increased when compared to that after 1 day of withdrawal (P < 0.01, respectively). Additionally, the expression of p-Akt-473 induced by CS was significantly increased when compared to those induced by CC not only after 1 day but also after 14 days of withdrawal (P < 0.05, P < 0.01, respectively). There were no differences in the expression of β-Actin across groups.

3.3 | Effects of LY294002 on heroin-seeking behavior and p-Akt levels in the NAc

Figure 3A revealed the effects of LY294002 on heroin-seeking behavior induced by a CS after 14 days of withdrawal. A two-way ANOVA revealed a significant main effect for dose of LY294002 ($F_{(2,\ 42)}$ = 35, P < 0.001), pokes ($F_{(1,\ 42)}$ = 321, P < 0.001), and a dose × pokes interaction ($F_{(2,\ 42)}$ = 32, P < 0.001). *Post-hoc* Bonferroni comparisons revealed a significant inhibition in the active pokes of the 2-mM LY294002 group or 5-mM LY294002 group (P < 0.01, respectively), as compared with the vehicle control group. There were no differences in the inactive pokes across the three groups.

Figure 3B revealed the p-Akt-473 levels in the NAc immediately after the rats completed the CS-induced heroin-seeking testing. A one-way ANOVA revealed a significant inhibition on the p-Akt-473

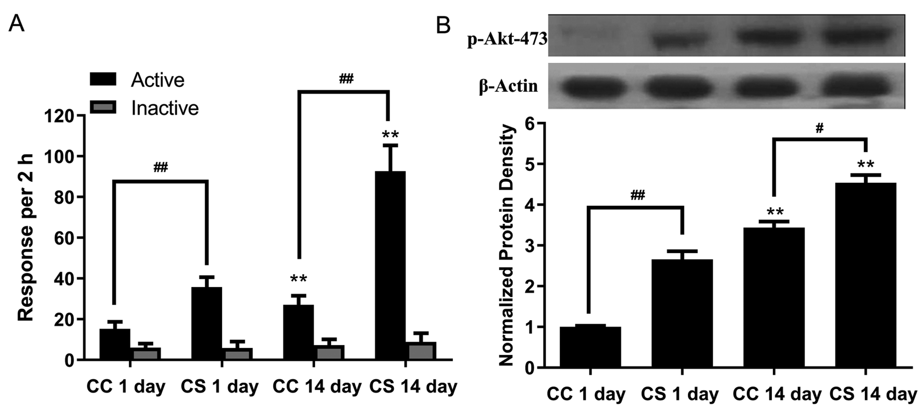

FIGURE 2 Effects of withdrawal period on the heroin-seeking behavior and expression of p-Akt-473 in the NAc. (A) Number of active or inactive nose-poke responses induced by a context cue (CC) or discrete conditioned cues (CS) after 1 day or 14 days of withdrawal. Data are expressed as mean ± SEM, n = 8 per group. (B) The insert immune-blot bands show representative changes in the expression of p-Akt-473 and β-Actin in the same NAc tissue sample. Histogram figures show the relative level of p-Akt-473 protein by densitometric quantification. Data are expressed as mean ± SEM, n = 3 per group. CC 1 day: exposure to CC following 1 day of withdrawal after heroin self-administration; CS 1 day: exposure to discrete CS following 1 day of withdrawal; CC 14 day: exposure to CC following 14 days of withdrawal; CS 14 day: exposure to discrete CS following 14 days of withdrawal. $^{**}P$ < 0.01, CC 14 days compared with CC 1 day or CS 14 days compared with CS 1 day. $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, CS 1 day compared with CC 1 day or CS 14 days compared with CC 14 days

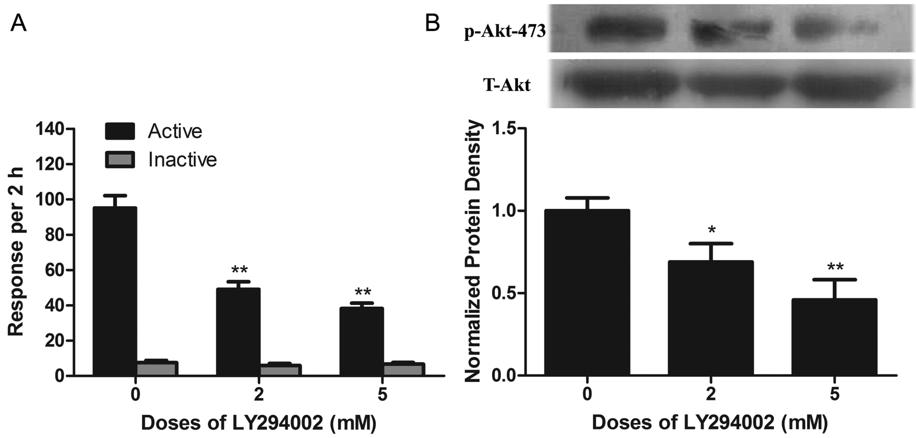

FIGURE 3 Effects of LY294002 on heroin-seeking behavior and p-Akt-473 levels in the NAc after 14 days of withdrawal. (A) Active or inactive responses induced by discrete conditioned cues in the rats microinjected with LY294002 at a dose of 2 or 5 mM in the NAc. Data are expressed as mean ± SEM, n = 8 per group. (B) The insert immune-blot bands show representative changes in the expression of p-Akt-473 and total Akt in the same NAc tissue sample. Histogram figures show the relative level of p-Akt-473 protein over the total Akt. Data are expressed as mean ± SEM, n = 3 per group. *P < 0.05, **P < 0.01, compared with the vehicle group

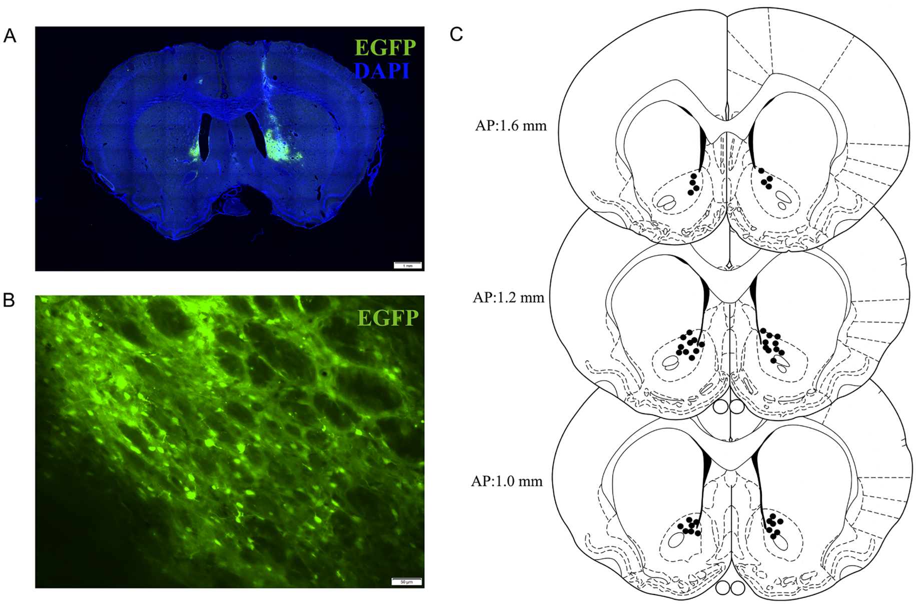

FIGURE 4 Effect of the expression of Akt1 siRNA lentivirus in the NAc. (A) Coronal section of the rat brain shows the microinjection sites and the expression of Akt1 lentivirus, as indicated by EGFP (green) protein under fluorescence microscopy. Scale bars = 1 mm. (B) The overlay magnification of NAc shows the neurons transfected with an Akt1 lentivirus. Scale bars = 50 μm. (C) Diagrams of coronal sections of the rat brain show the microinjection sites (NAc) of all rats

level after treatment with LY294002 ($F_{(2,6)}$ = 19.73, P < 0.01). *Post-hoc* Bonferroni comparisons revealed that the p-Akt-473 levels in the NAc were significantly inhibited after injection with 2-mM LY294002 (P < 0.05) or 5-mM LY294002 (P < 0.01), as compared with those in the control group. There were no differences in the expression of total-Akt across groups.

3.4 | GFP protein expression after Akt1 lentivirus microinjection and cannula placement sites in the NAc

Following the heroin-seeking test, the rats that had been micro-injected with a lentivirus were assessed for protein levels by western blot or histologically assessed for their levels of GFP protein expression. As illustrated in Figure 4A, a coronal section of the rat brain demonstrated the expression of GFP protein in the NAc. The overlay magnification of the NAc is illustrated in Figure 4B. All micro-injection sites in these rats are shown as diagrams of coronal sections of the rat brain in Figure 4C.

3.5 | Effects of β-arrestin 2 or Akt1 RNA interference on heroin-seeking behavior or p-Akt and p-CREB expression in the NAc

The effects of β-arrestin 2 or Akt1 RNA interference on heroin-seeking behavior induced by a CS after 14 days of withdrawal were shown in Figure 5A. A two-way ANOVA revealed a

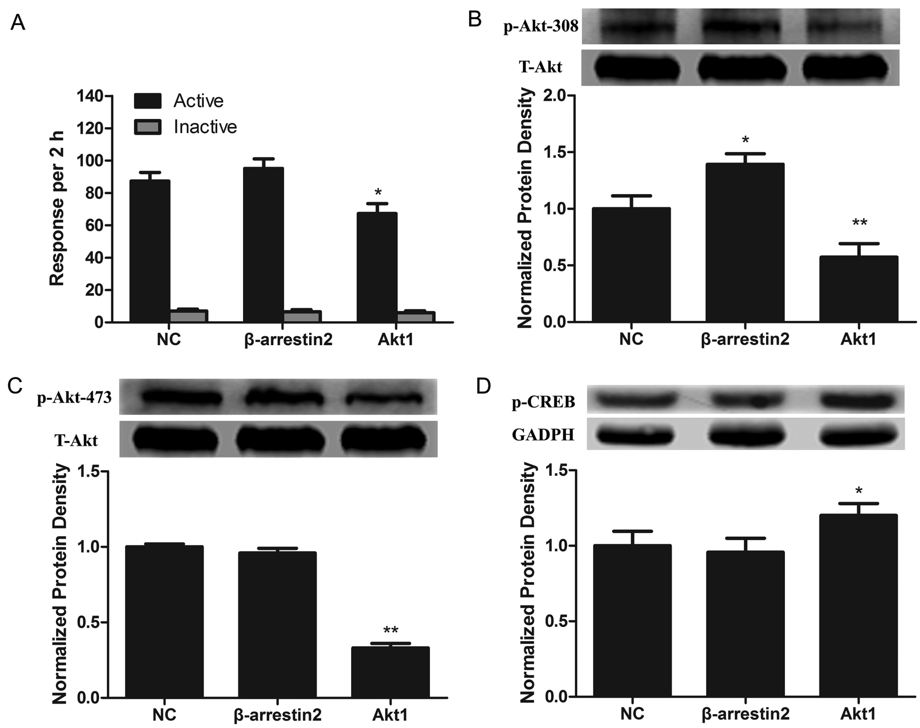

FIGURE 5 Effects of β-arrestin 2 or Akt1 RNA interference on the heroin-seeking behavior and protein expression in the NAc after 14 days of withdrawal. (A) Active or inactive responses induced by discrete conditioned cues in the rats microinjected with a negative control, β-arrestin 2, or Akt1 siRNA lentivirus. Data are expressed as mean ± SEM, $n = 7$ per group. (B) The representative changes in the expression of p-Akt-308 and total Akt, (C) p-Akt-473 and total Akt, and (D) p-CREB and GAPDH in the same NAc tissue sample are shown in the insert immune-blot bands. Histogram figures show the relative level of p-Akt-308 protein over total Akt, p-Akt-473 protein over total Akt, and p-CREB protein over GAPDH. Data are expressed as mean ± SEM, $n = 3$ per group. *$P < 0.05$, **$P < 0.01$, compared with the negative control (NC) group

significant treatment effect for treatment ($F_{(2, 36)} = 6.126$, $P < 0.01$), pokes ($F_{(1, 36)} = 497.5$, $P < 0.001$), and a treatment × pokes interaction ($F_{(2, 36)} = 5.514$, $P < 0.01$). *Post-hoc* Bonferroni comparisons revealed a significant inhibition in the active pokes of the Akt1 group ($P < 0.05$) compared to those of the NC group. However, β-arrestin 2 interference had no significant effect on the active pokes. There were no differences in the inactive pokes among groups.

Figure 5B showed the effects of β-arrestin 2 or Akt1 RNA interference on the protein expression levels of p-Akt-308 in the NAc. A one-way ANOVA revealed a significant treatment effect of RNA interference on p-Akt-308 levels ($F_{(2,6)} = 46.60$, $P < 0.001$). *Post-hoc* Bonferroni comparisons revealed that the protein expression of p-Akt-308 was increased after β-arrestin 2 interference ($P < 0.05$) but decreased after Akt1 interference compared to that of the NC group ($P < 0.01$). There were no differences in the total-Akt protein expression levels among those groups.

Figure 5C showed the effects of β-arrestin 2 or Akt1 RNA interference on p-Akt-473 protein expression levels in the NAc. A one-way ANOVA revealed a significant treatment effect of RNA interference on p-Akt-473 levels ($F_{(2,6)} = 254.2$, $P < 0.001$). *Post-hoc* Bonferroni comparisons revealed that the protein expression of p-Akt-473 was decreased after Akt1 interference ($P < 0.01$) but not after β-arrestin 2 interference when compared to that of the NC group. There were no differences in the total-Akt protein expression levels among those groups.

Figure 5D showed the effects of RNA interference on the protein expression of p-CREB in the NAc. A one-way ANOVA revealed a significant treatment effect of RNA interference on p-CREB levels ($F_{(2,6)} = 6.27$, $P < 0.05$). *Post-hoc* comparisons revealed a significant increasing effect on p-CREB levels after Akt1 interference ($P < 0.05$) but not after β-arrestin2 interference when compared to those of the NC group. There were no differences in the protein expression levels of GAPDH among groups.

4 | DISCUSSION

In the present study, we found that heroin-seeking behaviors induced by CC or CS were enhanced following a prolonged withdrawal period, reflecting the elevated levels of p-Akt-473 in the NAc. Microinjection of LY294002 significantly decreased CS-induced heroin-seeking behaviors and the expression of p-Akt-473 protein in the NAc after 14 days of withdrawal. Lentivirus-mediated Akt1 downregulation in the NAc also attenuated the cue-induced heroin-seeking behavior after 14 days of withdrawal. Meanwhile, p-Akt-308 and p-Akt-473 expression levels were all significantly decreased, while p-CREB expression was increased after Akt1 downregulation in the NAc. Lentivirus-mediated β-arrestin 2 knockdown increased the expression of p-Akt-308 in the NAc, without affecting heroin-seeking behavior. These data demonstrated that Akt phosphorylation in the NAc may be involved in the incubation of heroin-seeking and cue-induced heroin-seeking behavior.

Heroin-seeking behavior induced by CC or CS was more strongly enhanced after 14 days of withdrawal than that after 1 day of withdrawal. This is consistent with other studies that demonstrated a time-dependent increase in heroin seeking not only in the extinction test but also in the CS test which may include several extinction sessions prior to the test.[20,21] In our study, the CC reflects the extinction environment, while the CS-induced heroin-seeking test does not include the former extinction session. Thus, rats underwent spontaneous withdrawal but no extinction as the reinstatement procedure, and the CS was not exclusive to CC. The present results showed that heroin-seeking behaviors induced by CS were obviously stronger than those induced by CC, especially after a prolonged withdrawal period, indicating the incubation of heroin seeking over time.[22]

Akt activity varies in the different phases of drug addiction and has brain region specificity. The expression of p-Akt in the NAc increases after a single morphine injection but significantly decreases after chronic morphine administration.[7] In the striatum of amphetamine sensitization rats, the expression of p-Akt significantly increases 15 min after an amphetamine injection on the challenge day but decreases at 2 h after the amphetamine challenge.[23] Because amphetamine can stimulate Ca^{2+}/calmodulin-dependent kinase II (CaMKII) activity and CaMKII subsequently regulates the complex of PP2A and Akt, reduced Akt activity in response to amphetamine can be blocked by the treatment by CaMKII inhibition in culture cells.[24] Furthermore, reward memory formation in the basolateral amygdala-mPFc circuit involves a functional link between D1 receptors and extracellular signal-related kinase 1/2 (ERK1/2) signaling substrates in opiate-naïve state rats but switches to a D2R/Ca^{2+}/CaMKIIα-dependent memory substrate following chronic opiate exposure.[25] The "rapid" Akt activation in vitro or vivo may be mediated by dopamine D1 receptors and subsequently cAMP/PKA pathway, whereas the "late" Akt deactivation may be considered a result of dopamine D2 receptors that stimulate an Akt-β-arrestin-PP2A inactivation complex and underline the reward tolerance of drugs.[26] Our study found that the expression of p-Akt-473 in the NAc increased when rats were exposed to either a CC or CS after prolonged withdrawal, indicating that the activation of Akt signaling also occurred after prolonged withdrawal. Moreover, the enhancement of p-Akt-473 is positively related to the increase in heroin-seeking behaviors. These results suggest that the activation of Akt signaling in the NAc may contribute to the incubation of the heroin-seeking behavior after prolonged withdrawal.

In the present study, the inhibition of Akt activity by LY294002 decreased the CS-induced heroin-seeking behavior after prolonged withdrawal in a dose-dependent manner, alongside a decrease of p-Akt-473 in the NAc. Since previous other study has shown that intracerebroventricular microinjection with LY294002 did not affect mice locomotion activity.[27] The effect of LY294002 on heroin-seeking behavior and expression of p-Akt in the NAc did not account for the inhibitory action of locomotion. Thus, the results suggested that the PI3K/Akt signaling pathway may underlie the heroin-seeking behavior induced by CS. PI3K can be regulated by neurotrophic factors, one of the most important of them being BDNF. BDNF colocalizes with tyrosine hydroxylase in midbrain DA neurons and therefore can play a role in the synaptic plasticity of these neurons.[28] The downregulation of Akt/mTORC2 signaling pathway mediates the decrease in the size of VTA dopamine neurons and reward tolerance after chronic morphine.[8,29] This study shows that BDNF and TrkB in the VTA are downregulated in morphine-induced CPP.[30] The expression levels of BDNF in the NAc of rats significantly decrease after chronic heroin treatment, whereas BDNF expression is significantly increased in these rats when they undergo either naloxone-induced withdrawal or spontaneous withdrawal 1 day after the last heroin treatment.[31] However, this explanation is in conflict with that of another study that suggests that BDNF protein in the NAc is not involved in heroin incubation.[21] Several methodological issues should be considered when these discrepant results are interpreted. With regard to BDNF, one possibility is the difference in the molecular assay. Another result indicates that the BDNF serum levels in heroin-dependent patients are lower than those of healthy controls at baseline and increased after 26 weeks of abstinence.[32] Additionally, the intravenous injection of the inhibitor of TrkB, the receptor of BDNF, dose-dependently reduced the reinstatement of cocaine consumption in rats that were allowed either short or long access times to cocaine self-administration.[9] Increased Akt phosphorylation is observed in rat ventromedial PFC after 3 or 30 days of withdrawal from cocaine treatment; an intra-PFC infusion of the PI3K inhibitor wortmannin could reduce cocaine-seeking behaviors elicited by a cue.[33] Therefore, the upregulation of PI3K/Akt signaling pathway in our study may be mediated by the increased BDNF expression in the NAc, and the PI3K/Akt signaling pathway is particularly involved in CS-induced heroin seeking after prolonged withdrawal.

Akt1 is one of Akt isoforms and is ubiquitously expressed in tissues.[6] The downregulation of Akt1 by a lentivirus decreased p-Akt-308 and p-Akt-473 expression levels and increased p-CREB expression in the NAc. This is consistent with our previous results that demonstrated that the elevated expression of p-CREB in the NAc by an inhibitor of phosphodiesterase 4 could reduce CS-induced heroin-seeking behaviors after prolonged withdrawal.[34] The downregulation of β-arrestin 2 increased p-Akt-308 expression in the NAc but not p-Akt-473 or p-CREB. The ineffectiveness of β-arrestin 2 on heroin seeking was complex and may be due to a number of factors. First, tolerance to the behavioral effects of morphine is prevented by the downregulation of β-arrestin-2 in mice,[35] but opioid withdrawal at the cellular level in periaqueductal gray neurons is unaffected by β-arrestin 2 deletion.[36] Second, β-arrestin 2 can also interact with other signaling molecules, such as ERK. In addition, the dopamine D1 receptor-dependent β-arrestin 2/p-ERK signaling complex in the NAc mediates morphine-induced locomotor activity but not conditioned place preference.[37] Taken together, CS-induced heroin-seeking behavior after prolonged withdrawal might not be directly affected by the β-arrestin 2/Akt/PP2A signaling pathway.

In conclusion, the present results demonstrated that Akt and its phosphorylation in the NAc mediate the incubation of heroin-seeking behaviors after prolonged withdrawal, suggesting that Akt may thus serve as a potential target of therapies for the treatment of heroin relapse and addiction.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS CONTRIBUTION

HZ performed the experiments, analyzed the data, and wrote the manuscript. DZ, ZL, ML, FD, and QH performed the experiments. HL was responsible for the study concept and supervised the experiments. WZ was responsible for study design and critically revised the manuscript. All authors critically reviewed content and approved final version for publication.

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REFERENCES

1. Zhou W, Kalivas PW. N-acetylcysteine reduces extinction responding and induces enduring reductions in cue- and heroin-induced drug-seeking. *Biol Psychiatry*. 2008;63(3):338-340.
2. Shalev U, Morales M, Hope B, Yap J, Shaham Y. Time-dependent changes in extinction behavior and stress-induced reinstatement of drug seeking following withdrawal from heroin in rats. *Psychopharmacology (Berl)*. 2001;156(1):98-107.
3. Zhou W, Zhang F, Liu H, et al. Effects of training and withdrawal periods on heroin seeking induced by conditioned cue in an animal of model of relapse. *Psychopharmacology (Berl)*. 2008;203(4):677-684.
4. Pickens CL, Airavaara M, Theberge F, Fanous S, Hope BT, Shaham Y. Neurobiology of the incubation of drug craving. *Trends Neurosci*. 2011;34(8):411-420.
5. Franke TF, Yang S-I, Chan TO, et al. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*. 1995;81(5):727-736.
6. Hanada M, Feng J, Hemmings BA. Structure, regulation and function of PKB/AKT--a major therapeutic target. *Biochim Biophys Acta*. 2004;1697(1-2):3-16.
7. Muller DL, Unterwald EM. In vivo regulation of extracellular signal-regulated protein kinase (ERK) and protein kinase B (Akt) phosphorylation by acute and chronic morphine. *J Pharmacol Exp Ther*. 2004;310(2):774-782.
8. Russo SJ, Bolanos CA, Theobald DE, et al. IRS2-Akt pathway in midbrain dopamine neurons regulates behavioral and cellular responses to opiates. *Nat Neurosci*. 2007;10(1):93-99.
9. Verheij MM, Vendruscolo LF, Caffino L, et al. Systemic delivery of a brain-penetrant TrkB antagonist reduces cocaine self-administration and normalizes TrkB signaling in the nucleus accumbens and prefrontal cortex. *J Neurosci*. 2016;36(31):8149-8159.
10. Cahill ME, Bagot RC, Gancarz AM, et al. Bidirectional synaptic structural plasticity after chronic cocaine administration occurs through Rap1 small GTPase signaling. *Neuron*. 2016;89(3):566-582.
11. Chen G, Li T, Xiao J, et al. Ifenprodil attenuates methamphetamine-induced behavioral sensitization through the GluN2B-PP2A-AKT cascade in the dorsal striatum of mice. *Neurochem Res*. 2020;45(4):891–901.
12. Hudson R, Green M, Wright DJ, et al. Adolescent nicotine induces depressive and anxiogenic effects through ERK 1-2 and Akt-GSK-3 pathways and neuronal dysregulation in the nucleus accumbens. *Addict Biol*. 2020:e12891. https://doi.org/10.1111/adb.12891
13. Shi X, Miller JS, Harper LJ, Poole RL, Gould TJ, Unterwald EM. Reactivation of cocaine reward memory engages the Akt/GSK3/mTOR signaling pathway and can be disrupted by GSK3 inhibition. *Psychopharmacology (Berl)*. 2014;231(16):3109-3118.
14. Beaulieu JM, Sotnikova TD, Yao WD, et al. Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade. *Proc Natl Acad Sci U S A*. 2004;101(14):5099-5104.
15. Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR, Caron MG. An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell*. 2005;122(2):261-273.
16. Beaulieu JM, Gainetdinov RR, Caron MG. The Akt-GSK-3 signaling cascade in the actions of dopamine. *Trends Pharmacol Sci*. 2007;28(4):166-172.
17. Zhu H, Lai M, Chen W, et al. N-acetylaspartylglutamate inhibits heroin self-administration and heroin-seeking behaviors induced by cue or priming in rats. *Neurosci Bull*. 2017;33(4):396-404.
18. Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. 4th ed. Sydney: Academic Press; 1998.
19. Lai M, Zhu H, Sun A, et al. The phosphodiesterase-4 inhibitor rolipram attenuates heroin-seeking behavior induced by cues or heroin priming in rats. *Int J Neurosci*. 2014;17(09):1397-1407.
20. Airavaara M, Pickens CL, Stern AL, et al. Endogenous GDNF in ventral tegmental area and nucleus accumbens does not play a role in the incubation of heroin craving. *Addict Biol*. 2011;16(2):261-272.
21. Theberge FR, Pickens CL, Goldart E, et al. Association of time-dependent changes in mu opioid receptor mRNA, but not BDNF, TrkB, or MeCP2 mRNA and protein expression in the rat nucleus accumbens with incubation of heroin craving. *Psychopharmacology (Berl)*. 2012;224(4):559-571.
22. Grimm JW, Hope B, Wise RA, Shaham Y. Incubation of cocaine craving after withdrawal. *Nature*. 2001;412(6843):141-142.
23. Shi X, McGinty JF. Repeated amphetamine treatment increases phosphorylation of extracellular signal-regulated kinase, protein kinase B, and cyclase response element-binding protein in the rat striatum. *J Neurochem*. 2007;103(2):706-713.
24. Wei Y, Williams JM, Dipace C, et al. Dopamine transporter activity mediates amphetamine-induced inhibition of Akt through a Ca2+/calmodulin-dependent kinase II-dependent mechanism. *Mol Pharmacol*. 2007;71(3):835-842.
25. Rosen LG, Zunder J, Renard J, Fu J, Rushlow W, Laviolette SR. Opiate exposure state controls a D2-CaMKIIalpha-dependent memory switch in the amygdala-prefrontal cortical circuit. *Neuropsychopharmacology*. 2016;41(3):847-857.
26. McGinty JF, Shi XD, Schwendt M, Saylor A, Toda S. Regulation of psychostimulant-induced signaling and gene expression in the striatum. *J Neurochem*. 2008;104(6):1440-1449.
27. Deyama S, Ishikawa Y, Yoshikawa K, et al. Resolvin D1 and D2 reverse lipopolysaccharide-induced depression-like behaviors through the mTORC1 signaling pathway. *Int J Neuropsychopharmacol*. 2017;20(7):575-584.
28. Grimm JW, Lu L, Hayashi T, Hope B, Su T-P, Shaham Y. Time-dependent increases in brain-derived neurotrophic. *J Neurosci*. 2003;23(3):742-747.
29. Mazei-Robison MS, Koo JW, Friedman AK, et al. Role for mTOR signaling and neuronal activity in morphine-induced adaptations in ventral tegmental area dopamine neurons. *Neuron*. 2011;72(6):977-990.
30. Zhang H, Wang Q, Sun Q, et al. Effects of compound 511 on BDNF-TrkB signaling in the mice ventral tegmental area in morphine-induced conditioned place preference. *Cell Mol Neurobiol*. 2020. https://doi.org/10.1007/s10571-020-00848-9
31. Li Y, Xia B, Li R, Yin D, Wang Y, Liang W. Expression of brain-derived neurotrophic factors, neurotrophin-3, and neurotrophin-4 in the nucleus accumbens during heroin dependency and withdrawal. *Neuroreport*. 2017;28(11):654-660.

32. Zhang K, Jiang H, Zhang Q, Du J, Wang Y, Zhao M. Brain-derived neurotrophic factor serum levels in heroin-dependent patients after 26 weeks of withdrawal. *Compr Psychiatry*. 2016;65:150-155.
33. Szumlinski KK, Ary AW, Shin CB, et al. PI3K activation within ventromedial prefrontal cortex regulates the expression of drug-seeking in two rodent species. *Addict Biol*. 2018;24(6):1216-1226.
34. Sun A, Zhuang D, Zhu H, et al. Decrease of phosphorylated CREB and ERK in nucleus accumbens is associated with the incubation of heroin seeking induced by cues after withdrawal. *Neurosci Lett*. 2015;591:166-170.
35. Bu H, Liu X, Tian X, Yang H, Gao F. Enhancement of morphine analgesia and prevention of morphine tolerance by downregulation of beta-arrestin 2 with antigene RNAs in mice. *Int J Neurosci*. 2015;125(1):56-65.
36. Connor M, Bagley EE, Chieng BC, Christie BR. β-Arrestin-2 knockout prevents development of cellular μ-opioid receptor tolerance but does not affect opioid-withdrawal-related adaptations in single PAG neurons. *Br J Pharmacol*. 2015;172:492-500.
37. Urs NM, Daigle TL, Caron MG. A dopamine D1 receptor-dependent beta-arrestin signaling complex potentially regulates morphine-induced psychomotor activation but not reward in mice. *Neuropsychopharmacology*. 2011;36(3):551-558.

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